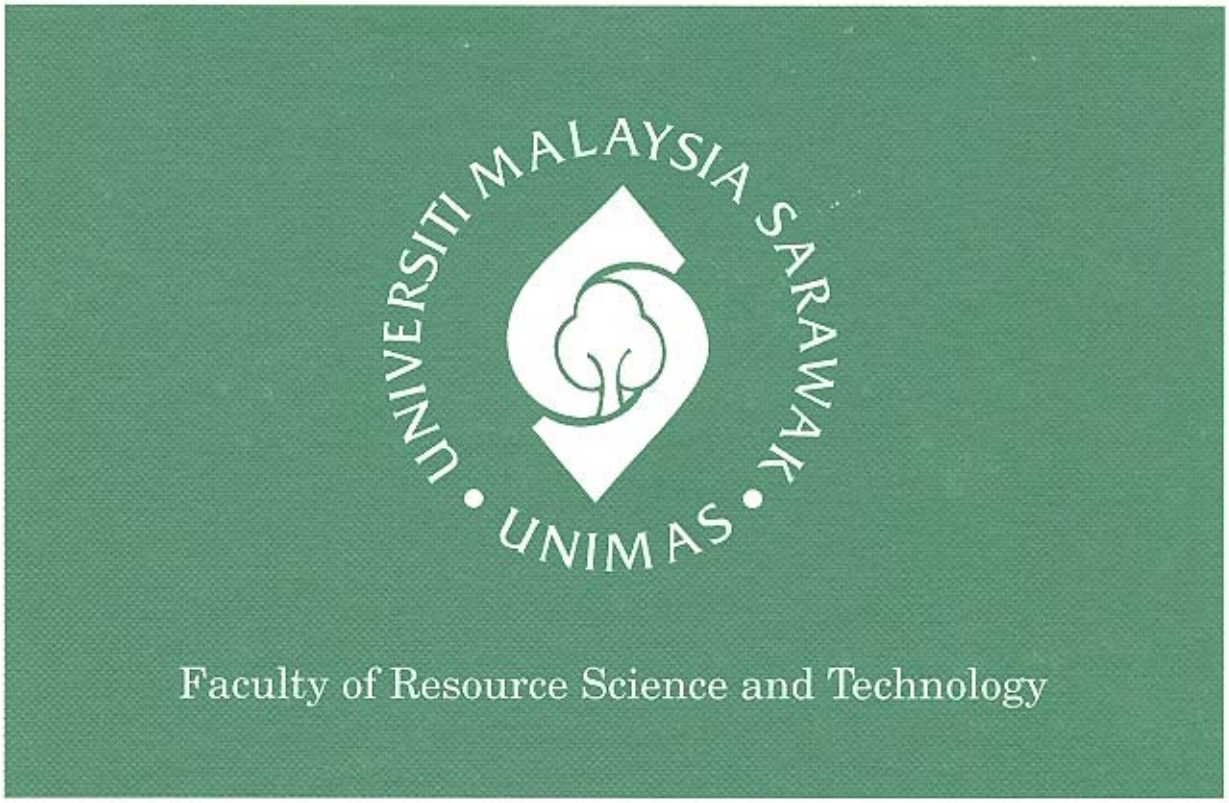

SCREENING OF *VIBRO SPP.* FROM RIVERINE ENVIRONMENTS USING MULTIPLEX PCR

Lawrance Anak Tuah

Bachelor of Science With Honours
(Resource Biotechnology)
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This project is submitted in partial fulfillment of
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Screening of *Vibrio spp.* from Riverine Environments Using Multiplex PCR

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ABSTRACT

Monthly screening for the presence of *Vibrio spp.* from environmental sources in Kuching, Sarawak was done using the polymerase chain reaction (PCR) technique. Nineteen samples were collected from different river sources each month during the six-month period (July-December, 2003). Multiplex PCR was used for simultaneous detection of three major genus of *Vibrio*; *Vibrio parahaemolyticus, Vibrio cholerae* and *Vibrio vulnificus* in a single tube PCR reaction. The three sets of primers *tl, ompW* and *vulCulsI* were used to amplify fragments of 450 bp, 588 bp and 222 bp in sizes for each species respectively. This application was useful for the rapid detection of the *Vibrio spp.* from water sources. The results indicated that *V. parahaemolyticus* was the dominant species occurring about 61% through out the sampling period, followed by *V. cholerae* (23%) and *V. vulnificus* (16%). This study showed that Multiplex PCR amplification was very specific, reduces labor and less time consuming and is useful for diagnostic laboratories to detect the presence of *Vibrio spp.* and also for epidemiological investigations.

Key words: Multiplex PCR , *Vibrio spp., Vibrio parahaemolyticus, Vibrio cholerae, Vibrio vulnificus.*

ABSTRACK

Penskrinan bulanan dilakukan untuk mengesan kehadiran Vibrio spp. daripada sumber persekitaran di Kuching, Sarawak menggunakan teknik Multiplex PCR. Sembilan belas sampel dikutip daripada sungai yang berlainan sepanjang enam bulan kajian (Julai-Disember, 2003). Teknik Multiplex PCR digunakan untuk mengesan kehadiran 3 jenis Vibrio spp. sekaligus dari sampel air sungai iaitu Vibrio parahaemolyticus, Vibrio cholerae dan Vibrio vulnificus di dalam satu tiub PCR. Tiga set primer digunakan iaitu tl, ompW dan vulCulsI yang bersaiz 450 bp, 588 bp dan 222 bp. Teknik ini didapati bertindakbalas secara positif mengesan kehadiran Vibrio spp. didalam air sungai. Keputusan menunjukkan V. parahaemolyticus adalah yang lebih dominan antara tiga Vibrio spp. sepanjang kajian dilakukan iaitu sebanyak 61%. V. cholerae pula sebanyak 23% manakala V. vulnificus sebanyak 16%. Daripada keputusan yang diperolehi menunjukkan teknik Multiplex PCR sesuai digunakan kerana spesifikasinya tepat pada gene sasaran sahaja, dapat mengurangkan tenaga kerja, menjimatkan masa dan boleh digunakan bahagian perubatan dan makmal rawatan sebagai langkah isyarat awal untuk mengesan kehadiran Vibrio spp.

Kata Kunci : Multiplex PCR , Vibrio spp., Vibrio parahaemolyticus, Vibrio cholerae, Vibrio vulnificus.

1. Introduction

Vibrio spp. are gram-negative, rod or curved shaped facultative anaerobes, halophilic and non-spore-forming bacteria. These bacteria are common organisms in temperate estuarine and saltwater environments (Janda *et al.*, 1988; Kaysner *et al.*, 1992; Joseph *et al.*, 1993). *Vibrio spp.* are resistant to alkaline environments but do not tolerate acidic conditions and will die rapidly in solution with pH lower than 6. In a previous study done by Alam *et al.* (2003), different ecological parameters such as nutrient, temperature and salinity influence the presence of *Vibrio spp.* in the environmental. Warmer temperature quickly increases the concentration of *Vibrio spp.* in both molluscan shellfish and seawater. However, fewer *Vibrio spp.* were cultured when temperature drop and salinity concentration low (Anon., 1999).

Several reports published stated that outbreaks of *Vibrio spp.* associated diseases present a public health problems in many countries, such as United States, India, Bangladesh, Canada, Africa, Latin America and Malaysia (Sarawak) (Anon. 2000; Micky *et al.*, 2000). Outbreak of *Vibrio spp.* related diseases are often associated with contaminated seafood or improperly cooked seafood especially raw seafood, such as raw oyster and also domestic use of highly polluted waters (Wilson and Moore, 1996; Depaola *et al.*, 1999). Most clinical symptoms of *Vibrio spp.* associated disease include diarrhea, vomiting, fever and muscle cramps (Depaola *et al.*, 1999; Mead *et al.*, 1999; Radu *et al.*, 1999). *Vibrio spp.* that are implicated in these cases are numerous. However, the three mostly publicized are *V. parahaemolyticus, V. cholerae* and *V. vulnificus.*

V. parahaemolyticus is a marine halophilic bacterium found naturally in seawater. Some strains of *V. parahaemolyticus* are pathogenic and has been involved in food poisoning in people consuming raw seafood such as oyster, crab and shrimps containing these strains.

Outbreaks of *V. parahaemolyticus* are also attributed to the consumption of contaminated seafood after cooking. This pathogen causes gastroenteritis and infection in human and has been associated with those strains with the ability to produce a thermostable direct hemolysin (*tdh*) (Honda and Iida, 1993; Jiang *et al.*, 1996; Depaola *et al.*, 2000; Anon. 2000).

V. cholerae is another member of the *Vibrio* family and it is distributed worldwide in the estuarine environment (Kaysner *et al.*, 1992; Joseph *et al.*, 1993). Pathogenic *V. cholerae*, such as *V. cholerae* O1, is an important agent that causes diarrhea in human. The outbreak of cholera is also associated with consumption of contaminated seafood, water and transmission from person-to-person. The virulence of *V. cholerae,* especially group 01, is different from other genus due to its ability to produce a cholera enterotoxin (*ctx*) that causes bloodless, watery diarrhea, voluminous watery stools, and muscle cramps (Radu *et al.*, 1999).

Like other members of the genus *Vibrio*, *V. vulnificus* is also found in the estuarine environment. This pathogen can cause disease to those who consume contaminated seafood. Furthermore, it can also cause an infection of the skin and septicemia when open wounds are exposed to warm seawater infested with this bacterium (Chuang *et al.*, 1992).

In Malaysia, Sarawak experienced a major cholera outbreak in Miri and the Northern Division from November 1997 to April 1998 (Micky *et al.*, 2000). According to the same study, contaminated water supplies from the surrounding rivers were the main mode of transmission for the outbreak which involved *V. cholerae* 01. During this outbreak more than 1000 cases were reported.

As generally known, Sarawak is blessed with numerous riverine environments, thus, there is a need to constantly monitor the distribution of *Vibrio spp.* in its rivers, especially its estuarine environment, where *Vibrio spp.* are generally found in abundance. One such monitoring tool for the detection of *Vibrio spp.* uses the polymerase chain reaction (PCR)-

based method. This method is proven to speed the detection of even non-culturable *Vibrio spp.* (Kaysner and DePaola 2001; Gonzalez-Rodriguez *et al.*, 2002).

Recently, researches have developed an alternative improvement to this technique such as Multiplex PCR. Multiplex PCR is a technique that uses multiple pairs of primers to amplify three or more target organisms simultaneously in a single reaction (Bej *et al.*, 1999; Chin-Ying *et al.*, 2003). Multiplex PCR has proven to be a rapid and highly sensitive method for specific detection of *Vibrio spp.* either from aquatic environments or seafood (Kong *et al.*, 2002; Chin-Ying *et al.*, 2003). This method is also commended due to its considerable saving of time and labour intensiveness, cost-effectiveness and have been performed as a relatively simple method to identify and differentiate *Vibrio spp.* (Bej *et al.*, 1999; Chin-Ying *et al.*, 2003).

In this research, the northern areas of Kuching were selected as sampling areas. Geographically, rivers in the northern part of Kuching are in close proximity to the sea such as the Sungai Bako, Sungai Jernang , Sungai Sejingkat, Sejingkat Causeway, Sungai Tabuan, Sungai Kuap and Sungai Melaban, where the intermixing of freshwater and saltwater is common. Developments along the rivers such as the construction of factories and the blooming of housing areas, as well as squatter areas may have aslo contributed to the microbiological pollution of the rivers. Sewage, rubbish and industrial wastes are often dumped directly into the rivers, which may be conducive to the existence and propagation of pathogenic microbes such as *Vibrio spp.*

The objective of this study is to perform monthly screenings of the presence of three common and potentially pathogenic *Vibrio spp.* namely *V. parahaemolyticus, V. cholera* and *V. vulnificus* from environmental sources in Kuching, Sarawak (Malaysia) using Multiplex PCR. All three *Vibrio spp.* isolated will be screened by targeting genes as *tl* for *V.*

parahaemolyticus, *ompW* for *V. cholera* and *VulCls1* for *V. vulnificus* (Taniguchi *et al.*, 1986; Lee *et al.*,1998; Bej *et al.*, 1999; Bisweswar *et al.*, 2000). The data collected from this study may contribute to the epidemiological studies on the mentioned *Vibrio spp.*

2. Methods and materials

2.1 Materials

Sterile bottle

Alkaline peptone water (APW), pH 8.5-8.6

Stock culture (LB broth + 20% glycerol)

LB broth + 2% NaCl

1X TBE buffer

10% Sodium docecyl sulfate

Proteinase K (Promega, USA)

Phenol / Choroform / Isoamyl alcohol (25:24:1)

3M K-Ac (pH4.5-5.5)

100% of Isopropanol

70% Cold ethanol

Template DNA

10X PCR reaction buffer (MWG, Germany)

5 pmol/µl primer *tl*-forward (MWG, Germany)

5 pmol/µl primer *tl*-reverse (MWG, Germany)

5 pmol/µl primer *ompW*-forward (MWG, Germany)

5 pmol/µl primer *ompW*-reverse (MWG, Germany)

5 pmol/µl primer *vulCls*-forward (MWG, Germany)

5pmol/µl primer *vulCls*-reverse (MWG, Germany)

25µM $MgCl_2$ (Promega, USA)

10μM dNTPs (Promega, USA)

Taq DNA polymerase (Promega, USA)

1.5% Agarose gel

DNA thermal cycler 2400 (Perkin Elmer)

Ethidium bromide (0.5μg/m)

Polaroid ™ Type 667 film

2.2 Collection of Samples

For this study, water samples were collected from highly populated areas in Kuching. The samples were collected from the following sites; Sungai Bako, Sungai Jernang, Sungai Sejingkat, Tambak Sejingkat, Sungai Tabuan, Sungai Kuap and Sungai Melaban. Two or three surface water samples were collected from each sampling sites, totaling to a number of 19 (Table 1) samples collected monthly during the 6 month period (July-December, 2003). Water samples were collected and stored at 4°C in pre-sterilized bottles, then transported to the laboratory and processed within 2 hours of collection.

Table 1: Location sampling site and number of sample collected during 6 month (July-December, 2003).

Sampling sites	Number of samples
Sg. Bako	3
Sg. Jernang	2
Sg. Sejingkat	2
Tambak Sejingkat	3
Sg. Tabuan	3
Sg. Kuap	3
Sg. Melaban	3
Total	19

2.3 Enrichment of water samples

The water samples were diluted to a 1: 5 ratio in alkaline peptone water (APW, pH 8.5-8.6) (Atlas, 1993). For each samples, 50 ml was added to 200 ml APW, mixed well and incubated at 37°C for 16-18 hours. Upon enrichment, 1.5 ml of the upper layer were used for bacterial DNA extraction. In addition 200 µl of the enriched homogenate were pipetted out from the upper layer and mixed with a mixture of LB broth with 15% glycerol as stock culture should immediate extraction was not performed.

2.4 Genomic DNA Isolation

Genomic DNA isolation was performed by modifying the procedure described by Ausubel and colleagues (1987). Approximately 1.5 ml of the enrichment were used for genomic DNA extraction. After centrifugation, cell pellets collected were resuspended in 700 µl of TE buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA] by vortexing. Next, 10 µl (w/v) of

10% SDS (sodium dodecyl sulphate) and 5 µl of 20 mg/ml Proteinase K (Promega, USA) was added and mix gently, incubated for 1 hour at 60°C. Then, 700 µl PCI (phenol-chloroform-isoamyl alcohol) was added, mixed gently and centrifuged at 12,000 rpm for 1 minute. The supernatant was then transferred in to a new 1.5 ml eppendorf tube. Next, 200 µl of 3 M K-Ac (pH 4.5-5.5) and 400 µl of cold isopropanol were added and mixed gently followed by centrifuge at 12,000 rpm for 10 minute. The DNA pellet was washed with 500 µl of 70% cold ethyl alcohol and centrifuges again at 7 minute. The supernatant was discarded and the DNA pellet was dried at room temperature. The DNA pellet was dissolved with 30 µl sterile distilled water and kept frozen at -20°C until further use.

2.5 Primer Used for Detection of Vibrio spp. Gene by Multiplex PCR

Table 2 below shows the properties of the specific primers, target genes, amplicon sizes, melting temperature and sources of gene sequences used for Multiplex PCR amplification in detecting the presence of all three *Vibrio spp.* All primer were obtained from MWG, Germany.

Table 2: Characteristics of the specific primers for the detection of *Vibrio spp.*

Vibrio spp.	Target gene	Primer sets	Tm (°C)	Amplicon Size (bp)	Source
V. parahaemolyticus	*tl-F*	5'- AAA GCG GAT TAT GCA GAA GCA CTG -3'	65	450	Bej *et al.*, 1999
	tl-R	5'- GCT ACT TTC TAG CAT CAT TTT CTC TGC-3'	63		
V. cholerae	*ompW-F*	5'- CAC CAA GAA GGT GAC TTT ATT GTG-3'	63	588	Bisweswar *et al.*, 2000
	ompW-R	5'- GAA CTT ATA ACC ACC CGC G-3'	53		
V. vulnificus	*vulCisl-F*	5'- GCT ATT TCA CCG CCG CTC AC-3'	59	222	Lee *et al.*, 1998
	vulCisl-R	5'- CCG CAG AGC CGT AAA CCG AA-3'	59		

Notes: bp = base pair
tl = thermolabile hemolysin
ompW = outer membrane protein
vulCisl = hemolysin /cytolysin gene

2.6 Multiplex PCR reaction and Cycling Condition

Multiplex PCR amplification was run in a 25 µl final volume. The mixture Master-mix was prepared consisting of 2.5 µl of 10X PCR reaction buffer, 1.0 µl of each of the 5 pmol/µl forward and reverse primers for *tl*, *ompW* and *Vulcls* , 0.5 µl of 10 µM dNTPs, 1.5 µl of 25 µM $MgCl_2$, 0.3 µl of 5 unit/µl Taq DNA polymerase and 9.2 µl volume of sterile distilled water and 20 µl of the Master-mix was aliquoted into 0.2 µl PCR tubes. Then 5 µl of DNA template was pipetted into the respective reaction tubes.

Table 3: List of the Multiplex PCR reaction used in this study.

Template DNA	= 5.0 µl
10X PCR reaction buffer	= 2.5 µl
5 pmol/ml primer *tl*-F	= 1.0 µl
5 pmol/ml primer *tl*-R	= 1.0 µl
5 pmol/ml primer *ompW*-F	= 1.0 µl
5 pmol/ml primer *ompW* –R	= 1.0 µl
5 pmol/ml primer *Vulcls* –F	= 1.0 µl
5 pmol/ml primer *Vulcls*-R	= 1.0 µl
25 mM $MgCl_2$	= 2.0 µl
10 mM dNTPs	= 0.5 µl
1 u/ml Taq DNA polymerase	= 0.3 µl
Sterile distilled water	= 8.7 µl
Total	= 25.0 µl

Multiplex PCR reaction was run in a DNA thermal cycler 2400 (Perkin Elmer), by setting the following temperature-cycling parameters : initial denaturation at 94°C for 3 minute; then 30 cycles of amplification of each cycles consisted of denaturation at 94°C for 1 minute, primer annealing at 50°C for 1 minute and primer extension at 72°C for 2 minute.

Following the amplification cycles, samples were kept at 72°C for 7 minute to allow final extension of incompletely DNA synthesized.

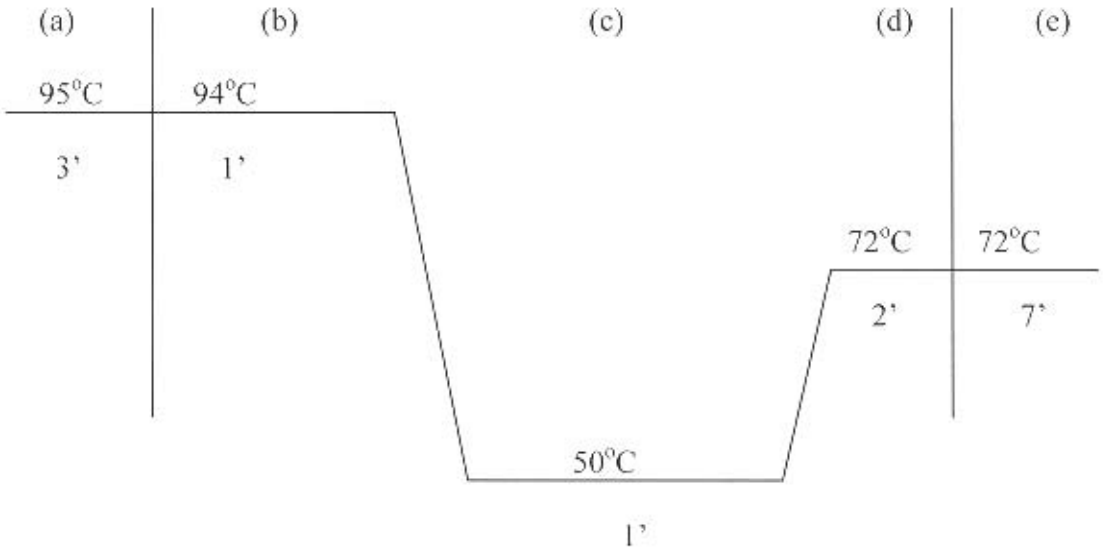

Figure 1: PCR machine cycling parameter; (a) initial denaturation, (b) denaturation, (c) annealing temperature, (d) extension and (e) final extension.

2.7 Visualization of PCR products

All PCR products were separated in a 1.7% (w/v) agarose gel electrophoresis. Electrophoresis was performed using 1X TBE buffer and a constant voltage of 70 V at 2 hour. Then, the gel was stained with ethidium bromide for 15 minute and visualized on a UV transilluminator (Fotodyne, WI) and photographed with Polaroid ™ Type 667 film.

3.0 Results

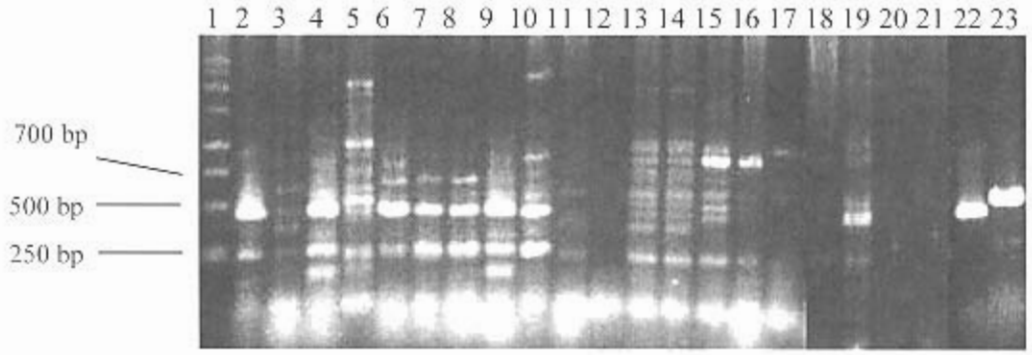

Figure 2: Agarose gel electrophoresis showing the results from PCR amplification product from samples collected in July. Lane 1, 1kb ladder; lane 2,3,4, Sungai Bako; lane 5,6, Sungai Jenang; lane 7,8, Sungai Sejingkat; lane 9,10,11, Tambak Sejingkat; lane 12,13,14, Sungai Tabuan; lane 15,16,17, Sungai Kuap; lane 18,19,20, Sungai Melaban; lane 21, negative control; lane 22, positive control VP28; lane 23, positive control SC4.

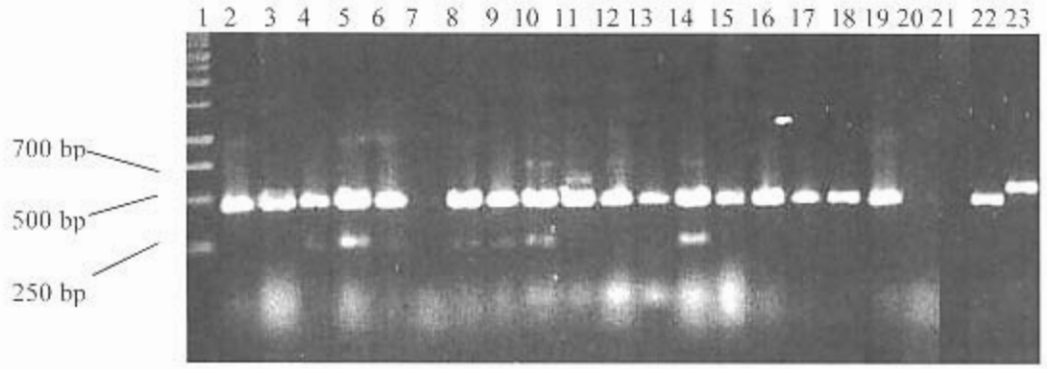

Figure 3: Agarose gel electrophoresis showing the results from the PCR amplification product from samples collected in August. Lane 1, 1kb ladder; lane 2,3,4, Sungai Bako; lane 5,6, Sungai Jenang; lane 7,8, Sungai Sejingkat; lane 9,10,11, Tambak Sejingkat; lane 12,13,14, Sungai Tabuan; lane 15,16,17, Sungai Kuap; lane 18,19,20, Sungai Melaban; lane 21, negative control; lane 22, positive control VP28; lane 23, positive control SC4.

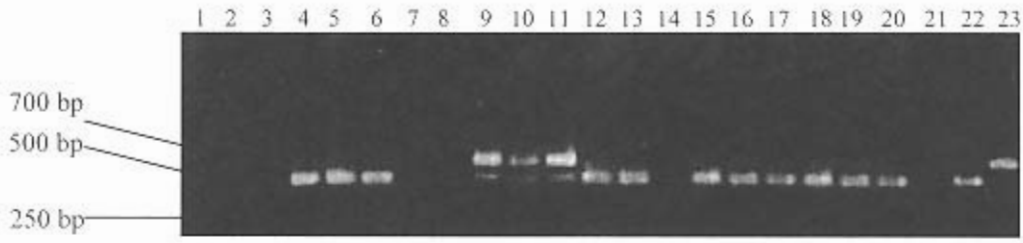

Figure 4: Agarose gel electrophoresis showing the results from PCR amplification product from samples collected in September. Lane 1, 1kb ladder; lane 2,3,4, Sungai Bako; lane 5,6, Sungai Jernang; lane 7,8, Sungai Sejingkat; lane 9,10,11, Tambak Sejingkat; lane 12,13,14, Sungai Tabuan; lane 15,16,17, Sungai Kuap; lane 18,19,20, Sungai Melaban; lane 21, negative control; lane 22, positive control VP28; lane 23, positive control SC4.

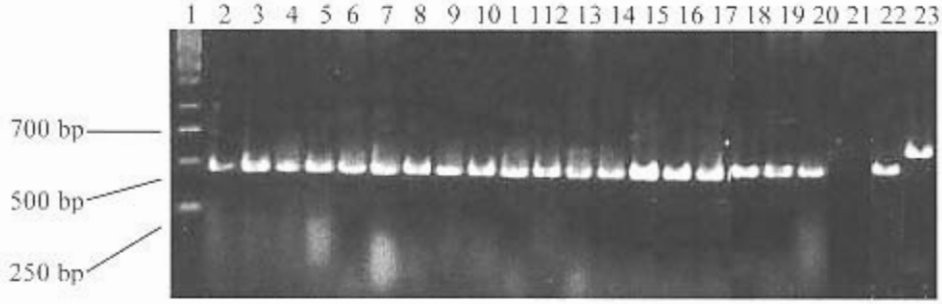

Figure 5: Agarose gel electrophoresis showing the results from PCR amplification product from samples collected in October. Lane 1, 1kb ladder; lane 2,3,4, Sungai Bako; lane 5,6, Sungai Jenang; lane 7,8, Sungai Sejingkat; lane 9,10,11, Tambak Sejingkat; lane 12,13,14, Sungai Tabuan; lane 15,16,17, Sungai Kuap; lane 18,19,20, Sungai Melaban; lane 21, negative control; lane 22, positive control VP28; lane 23, positive control SC4.

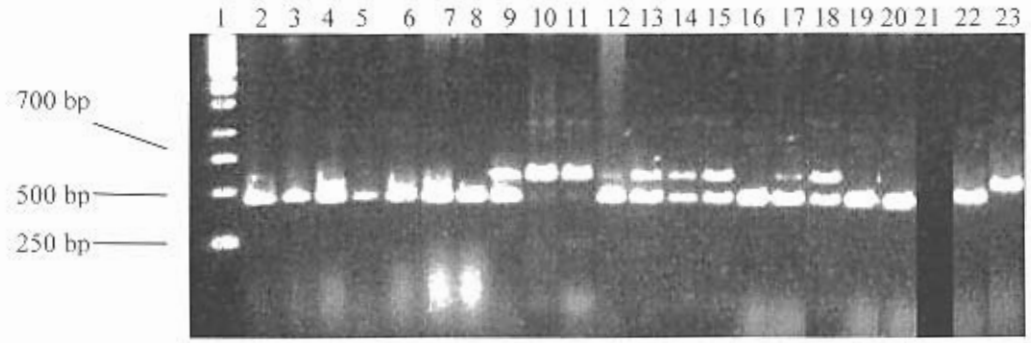

Figure 6: Agarose gel electrophoresis showing the results from PCR amplification product from samples collected in November. Lane 1, 1kb ladder; lane 2,3,4, Sungai Bako; lane 5,6, Sungai Jenang; lane 7,8, Sungai Sejingkat; lane 9,10,11, Tambak Sejingkat; lane 12,13,14, Sungai Tabuan; lane 15,16,17, Sungai Kuap; lane 18,19,20, Sungai Melaban; lane 21, negative control; lane 22, positive control VP28; lane 23, positive control SC4.

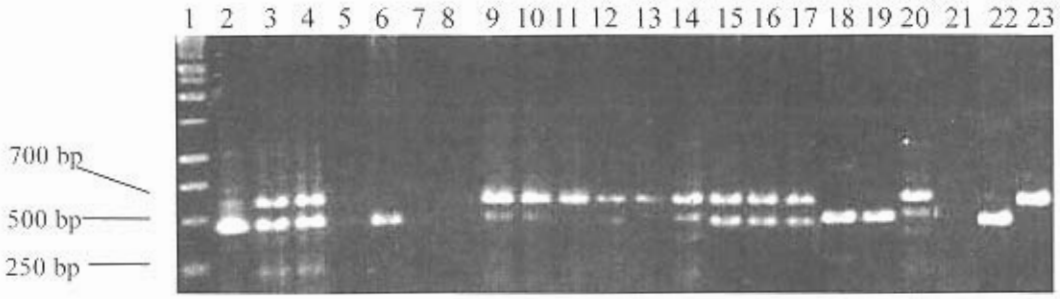

Figure 7: Agarose gel electrophoresis showing the results from PCR amplification product from samples collected in December. Lane 1, 1kb ladder; lane 2,3,4, Sungai Bako; lane 5,6, Sungai Jenang; lane 7,8, Sungai Sejingkat; lane 9,10,11, Tambak Sejingkat; lane 12,13,14, Sungai Tabuan; lane 15,16,17, Sungai Kuap; lane 18,19,20, Sungai Melaban; lane 21, negative control; lane 22, positive control VP28; lane 23, positive control SC4.

Table 4: List of *Vibrio spp.* data collected from July-December, 2003.

			Jul	Aug	Sept	Oct	Nov	Dec
Sg. Bako	SB1	Vp	+	+	–	+	+	+
		Vc	–	–	–	–	–	+
		Vv	+	–	–	–	–	–
	SB2	Vp	+	+	–	+	+	+
		Vc	+	–	–	–	–	+
		Vv	–	–	–	–	–	+
	SB3	Vp	+	+	+	+	+	+
		Vc	–	–	–	–	–	+
		Vv	+	+	–	–	–	+
Sg. Jernang	SJ1	Vp	–	+	+	+	+	–
		Vc	+	–	–	–	–	–
		Vv	+	+	–	–	–	–
	SJ2	Vp	+	+	+	+	+	+
		Vc	–	–	–	–	–	–
		Vv	+	+	–	–	–	–
Sg. Sejinkat	SS1	Vp	+	–	–	+	+	–
		Vc	+	–	–	–	–	–
		Vv	+	–	–	–	–	–
	SS2	Vp	+	+	+	+	+	–
		Vc	–	–	–	–	–	–
		Vv	+	+	–	–	–	–
T. Sejingkat	TS1	Vp	+	+	+	+	+	+
		Vc	+	–	+	–	+	+
		Vv	+	+	–	–	–	–
	TS2	Vp	+	+	+	+	–	+
		Vc	+	–	+	–	+	+
		Vv	+	+	–	–	–	–
	TS3	Vp	–	+	+	+	–	–
		Vc	–	+	+	–	+	+
		Vv	+	–	–	–	–	–
Sg. Tabuan	ST1	Vp	+	+	+	+	+	+
		Vc	–	–	–	–	+	+
		Vv	+	–	–	–	–	–
	ST2	Vp	–	+	+	+	+	–
		Vc	+	–	–	–	+	+
		Vv	–	–	–	–	–	–
	ST3	Vp	+	+	–	+	+	+
		Vc	+	–	–	–	+	+
		Vv	+	+	–	–	–	–
Sg. Kuap	SK1	Vp	+	+	+	+	+	+
		Vc	+	–	–	–	+	+
		Vv	+	–	–	–	–	–
	SK2	Vp	+	+	+	+	+	+
		Vc	–	–	–	–	–	+
		Vv	+	–	–	–	–	–
	SK3	Vp	–	+	+	+	+	+
		Vc	–	–	–	–	+	+
		Vv	–	–	–	–	–	–
Sg. Melaban	SM1	Vp	–	+	+	+	+	+
		Vc	+	–	–	–	+	–
		Vv	+	–	–	–	–	+
	SM2	Vp	+	+	+	+	+	+
		Vc	+	–	–	–	–	–
		Vv	+	–	–	–	–	–
	SM3	Vp	–	–	+	+	+	+
		Vc	–	–	–	–	–	+
		Vv	–	–	–	–	–	–

Notes: + = presence
– = absence
Vp = *V. parahaemolyticus*
Vc = *V. cholerae*
Vv = *V. vulnificus*

Table 5: Overall number and prevalence of *Vibrio spp.* samples from July-December, 2003.

	V. parahaemolyticus	*V. cholerae*	*V. vulnificus*
July	13	10	15
August	17	1	7
September	15	3	0
October	19	0	0
November	17	9	0
December	14	13	3
Total	95	36	25
Percentages of prevalence in rivers (%) (July-Dec, 2003)	83	32	22
Percentages of prevalence among *Vibrio spp* (%).	61	23	16

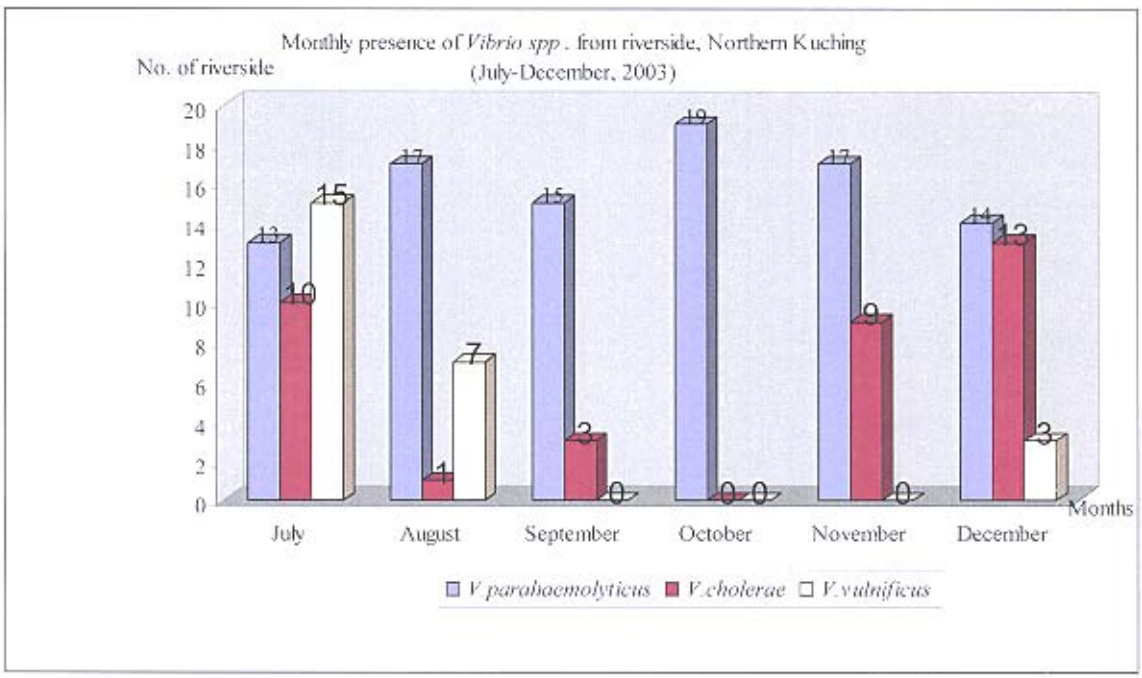

Figure 7: Monthly distribution of *Vibrio spp.* from sample sites (July-December, 2003).

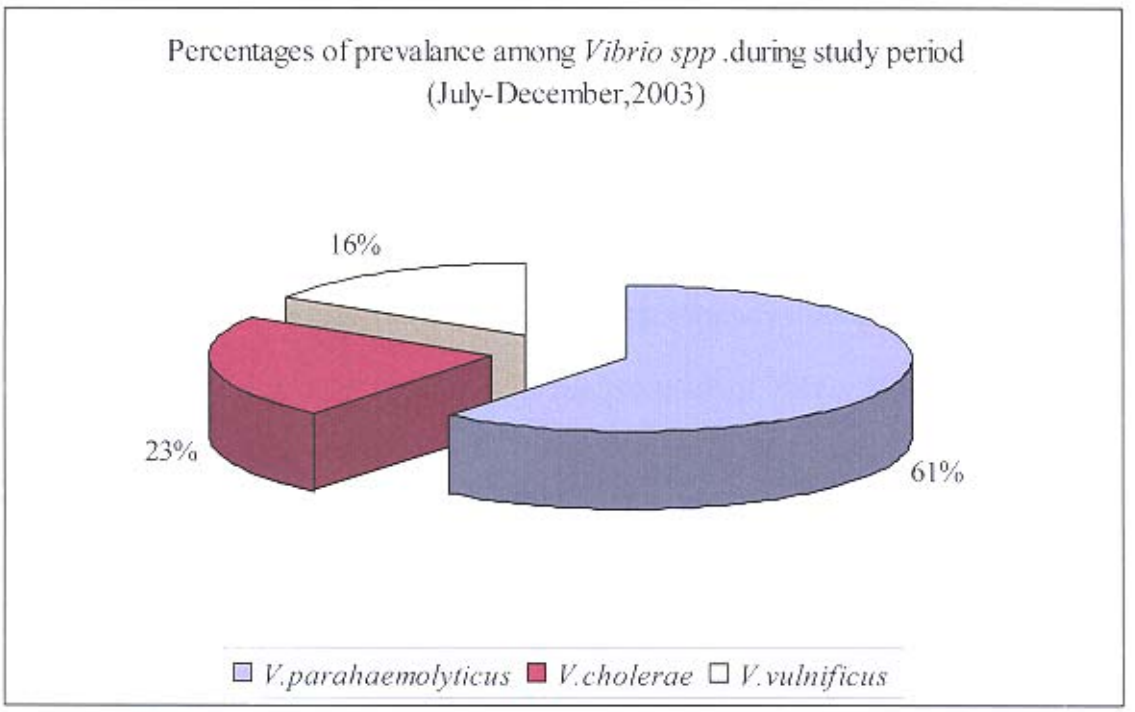

Figure 8: Pie chart showing prevalance percentages of among *Vibrio spp*. (July-December, 2003).

4.0 Discussion

A total of 114 water samples were collected and processed from seven rivers in Kuching during the period of study (July-Dec, 2003). The results indicated that the majority of the water samples collected from each river during this study were positive for the presence for *V. parahaemolyticus*. The frequency of the presence of *Vibrio spp.* in different waters sources is shown in Table 5.

In July, 15 of the surface water samples collected showed positive result for *V. vulnificus* from SB1, SB3, SJ1, SJ2, SS1, SS2, TS1, TS2, TS3, ST1, ST3, SK1, SK2, SM1 and SM2, 13 samples showed positive the presence of *V. parahaemolyticus* from SB1, SB2, SB3, SJ2, SS1, SS2, TS1, TS2, ST2, ST3, SK1, SK2 and SM2 while 10 samples showed positive result for *V. cholerae* from SB2, SJ1, SS1, TS1, TS2, ST2, ST3, SM1 and SM2 (Figure 2).

In August, 17 samples were positive for the presence of *V. parahaemolyticus* (SB1, SB2, SB3, SJ1, SJ2, SS2, TS1, TS2, TS3, ST1, ST2, ST3, SK1, SK2, SK3, SM1 and SM2), 7 samples showed positive result for *V. vulnificus* (SB3, SJ1, SJ2, SS2, TS1, TS2 and ST3) but only one sample showed positive result for *V. cholerae* (TS3) (Figure 3). Futhermore, 15 samples collected in September were positive for the presence of *V. parahaemolyticus* (SB3, SJ1, SJ2, SS2, TS1, TS2, TS3, ST1, ST2, SK1, SK2, SK3, SM1, SM2 and SM3), while 3 samples were positive for *V. cholerae* (TS1, TS2 and TS3). However, all 19 samples collected in September were negative for the presence of *V. vulnificus* (Figure 4). In October, all 19 samples were positive for the amplification of the *tl* gene indicating the presence of *V. parahaemolyticus* but none were positive for *V. cholerae* and *V. vulnificus* (Figure 5). Nineteen samples processed in November also showed that almost all samples (17 samples)

were positive for the presence of *V. parahaemolyticus* (SB1,SB2, SB3, SB3, SJ1, SJ2, SS1, SS2, TS1, ST1, ST2, ST3, SK1, SK2, SK3, SM1, SM2 and SM3) with 9 samples being positive for the presence of *V. cholerae* from TS1, TS2, TS3, ST1, ST2, ST3, SK1, SK3 and SM1 but none samples were positive for the presence of *V. vulnificus* (Figure 6).

In December, all three *Vibrio spp.* were present from the river water samples. The majority of the samples showed positive result for the presence of *V. parahaemolyticus* (14) (SB1, SB2, SB3, SB3, SJ2, TS1, TS2, ST1, ST3, SK1, SK2, SK3, SM1, SM2 and SM3), followed by 13 positive results for *V. cholerae* (SB1, SB2, SB3, SB3, TS1, TS2, ST1, ST2, ST3, SK1, SK2, SK3 and SM3) and three samples were positive the presence of *V. vulnificus* (SB2, SB3 and SM1).

From general observations done, there is a correlation between the changes of weather with the presence of several *Vibrio spp.* in the river samples. During the dryer months of July and August, the datas collected showed the presence of almost all three *Vibrio spp.* Dryer weather meant increased level of salinity which is conclusive to the growth of the three bacterial species. Interestingly, the presence of certain *Vibrio spp.* in the rainy month differed from the dryer month. Most of water samples collected during the rainy season (September-October) indicated the presence of *V. parahaemolyticus* and *V. cholerae*. But none of it indicated the presence of *V. vulnificus*. From Table 5, samples from the month of September, October, November, and December showed the presence of *V. parahaemolyticus* to be at 70%-90%, with 15% and 68% of water samples collected in September and December being positive for the presence of *V. cholerae* (Figure 3 and 6). This may lead to the deduction that *V. parahaemolyticus* and *V. cholerae* are more prevalent and dominant during rainy season. Theoretically, during the rainy season, water salinity is lower. Unfortunately, the research did not have the opportunity to record the water salinity level during the course of the study.

Datas collected showed that the river with the highest prevalence of the three *Vibrio spp.* was Tambak Sejingkat, Sungai Tabuan and Sungai Kuap.

There have been several reports of studies being developed using PCR-based method to directly detect only single strain of *Vibrio spp.* (Koch *et al.*, 1993; Lee *et al.*, 1993; Blackstone *et al.*, 2003). This method could be applied even when small sample are to be analyzed. But more recently, previous studies have developed alternative approaches to detect simultaneously the presence of several *Vibrio spp.* and other pathogenic bacteria using Multiplex PCR from seafood and environmental sources. Bej *et al.*, (1999) used Multiplex PCR to detect several genes of *V. parahaemolyticus* in shellfish. These methods were rapid, highly sensitive, quicker and easier when targeting three gene segments simultaneously and enabled the detection of total and hemolysin producing *V. parahaemolyticus*. Ching *et al.*, (2003) also published a report on the use of specific targets for the detection of pathogenic bacteria in shellfish by Multiplex PCR, namely *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus* and *Salmonella enterica* serotype strain, the *vvh*, *tl*, *ctx* and *spvB* genes in single tube PCR followed by Covalink™ microwell plate sandwich hybridization. The result of their investigation indicated the positive identification of these bacteria in seafood. These virulence and non-virulence genes (*vvh*, *tl*, *ctx* and *spvB*) can be used as specific markers for PCR-based method of detection for these pathogens. However, none of research groups used Multiplex PCR method for rapid detections of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* from water or environment samples. We have successfully used the Multiplex PCR method to detect these three species simultaneously.

In this study, several mixture of genomic DNA were used as DNA templates for Multiplex PCR amplification to screen for the presence of *Vibrio spp.* Reactions were performed in one-tube PCR mixtures utilizing the use of 3 primer pairs (*tl*, *ompW* and

vulCulsI). These sets of primers were specific for the detection of the respective individual *Vibrio spp.* such as *V. parahaemolyticus, V. cholerae* and *V. vulnificus*, targeting the *tl, ompW* and *vulCulsI* genes which were 588 bp, 450 bp and 222 bp in sizes respectively.

The major challenge when using Multiplex PCR is the presence of many PCR primers in a single tube that can cause several problems. Among these are the formation of multiple mismatching of primers, primer-dimer and other nonspecific products that may interfere with the amplification of specific products. To avoid the mismatching of the primers, all primers were pair analyzed against each other using the BlastN program from the GenBank database to rule out cross species priming. Computer analyses confirmed that all the oligonucleotide primer pairs showed significant affinities only for their target species and non-mismatch were obtained from the primers used. To minimize the problem of PCR product interpretation, the sizes of the amplicons were initially fixed to be different from each other in sizes making size discrimination easier by using gel electrophoresis (Table 2) (Kong *et al.*, 2002). In addition, another challenge presented by the used of Multiplex PCR method were the present of PCR inhibitors and proteins in the extracted DNA. To overcome this problem, organic purification may be done several times.

In this analysis, the lowest annealing temperatures of 53°C from the three pairs of primers were used (Table 2). However, this temperature failed to produce any amplification as shown by gel electrophoresis. To rectify this problem, the temperatures were reduced to 50°C for 1 minute for the best annealing temperature and the initial denaturing temperature was increased to 95°C for 3 minutes to minimize the incidences of non-specific banding. The volume of 25 µM $MgCl_2$ and extension time on the Multiplex PCR also affected the PCR amplifications. The volume of $MgCl_2$ was increased from 1.5 µl to 2.0 µl as this volume produces better intensity of PCR products. As shown in Figure 1, the optimum extension time

was 72°C at 2 minute for initial extension and 72°C for 7 minute of final extension. Furthermore, optimization also included the amount of template DNA used. The amplification of the Multiplex PCR product harboured unspecific bands when 7.0 µl template DNA was used (Figure 2). On the other hand, when the amount of the templates DNA was reduced to 5.0 µl unspecific bands were eliminated.

When a large number of samples for screening purposes are processed to analyze individual species, the analyses may lead to longer wait and higher cost for identification and confirmation. Thus, identification of *Vibrio spp.* using Multiplex PCR with three primer or more pairs of primers in the same reaction volume reduces labor, saves time and is ultimately economically sound. In this experiment, 19 samples can be processed within two days to obtain result after sampling, processing and enrichment. These suggest that the Multiplex PCR method of identification compared with basic PCR technique is useful for diagnostic laboratories to detect *Vibrio spp.* and also for epidemiological investigations.